

Temperature regulates NF- κ B dynamics and function through timing of A20 transcription

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NF- κ B signaling plays a pivotal role in control of the inflammatory response. We investigated how the dynamics and function of NF- κ B were affected by temperature within the mammalian physiological range (34 °C to 40 °C). An increase in temperature led to an increase in NF- κ B nuclear/cytoplasmic oscillation frequency following Tumor Necrosis Factor alpha (TNF α) stimulation. Mathematical modeling suggested that this temperature sensitivity might be due to an A20-dependent mechanism, and A20 silencing removed the sensitivity to increased temperature. The timing of the early response of a key set of NF- κ B target genes showed strong temperature dependence. The cytokine-induced expression of many (but not all) later genes was insensitive to temperature change (suggesting that they might be functionally temperature-compensated). Moreover, a set of temperature- and TNF α -regulated genes were implicated in NF- κ B cross-talk with key cell-fate-controlling pathways. In conclusion, NF- κ B dynamics and target gene expression are modulated by temperature and can accurately transmit multidimensional information to control inflammation.

NF- κ B | temperature | A20 | transcription | dynamics

The nuclear factor kappa B (NF- κ B) signaling pathway is a key mediator of the immune system, which regulates cell fate as well as stress and inflammatory responses. It responds to diverse stimuli, including viral and bacterial pathogens, free radicals, cytokines, and growth factors (1), and can process information from multiple input signals to regulate the transcription of >500 genes (1, 2). In response to persistent cytokine signaling, the NF- κ B transcription factor complex (typically RelA/p65:p50) can oscillate repeatedly between the cytoplasm and nucleus with a period of ~100 min (3–5). This is regulated by a set of negative feedback loops, notably via the genes expressing I κ B α , I κ B β , and A20 proteins. The dynamics, and particularly frequency, of NF- κ B oscillations may be one component that regulates the pattern of target gene expression and the physiological response (3, 6–8).

Changes in temperature alter the rates of all biological-chemical reactions. The ability of mammals to regulate temperature is an important aspect of survival. In humans, there are natural physiological temperature changes involved in day–night (36.4 °C to 37.5 °C) (9), menstrual (~0.5 °C increase after ovulation) (10), and (more variable) seasonal temperature cycles, as well as between peripheral tissues (~34 °C) and core body temperature (~37 °C). Temperature also increases with fever (typically 37 °C to 40 °C). The direct functional consequences of these temperature changes are largely unclear, but there has been a growing body of evidence that the efficacy of the immune response may be modulated by temperature (11, 12).

Despite the long-established associations between temperature, fever, and inflammation, as well as between inflammation and NF- κ B signaling, it remains unknown how temperature affects the NF- κ B system. We investigated how temperature change in the physiological range affects the NF- κ B system response,

by measuring both the oscillatory dynamics and the downstream gene expression response.

Results

To investigate the effect of temperature on NF- κ B dynamics, we used time-lapse live-cell microscopy to study cytoplasmic to nuclear oscillations of NF- κ B across the physiological temperature range, 34 °C to 40 °C. Bacterial artificial chromosomes expressing human RelA and I κ B α fused to fluorescent proteins were generated to enable detection of both the dynamics of RelA translocations and I κ B α degradation and resynthesis, allowing expression at more physiological levels. The RelA and I κ B dynamics were observed to have regular opposite phases (Fig. 1*A* and *B*). A rapid temperature shift from 37 °C to 40 °C caused an immediate decrease in period of RelA translocation from ~100 min to ~74 min in TNF α -treated (10 ng/mL), transiently

Significance

Inflammation is often accompanied by temperature change, but little is known about the role of temperature in the inflammatory response. We show that physiologically relevant temperature changes significantly perturb NF- κ B dynamics following TNF α stimulation in single cells. Using experimentation informed by mathematical modeling, we found that these changes were mediated, at least in part, through the key feedback gene TNFAIP3/A20. Curtailing A20 expression removed temperature sensitivity across the fever range (37 °C to 40 °C). Gene expression was generally unaffected between these temperatures, although a select set of NF- κ B-regulated genes was up-regulated at early time points. These genes were predominantly involved in inflammation, signaling, and cell fate. The cellular response to inflammation may therefore be mechanistically and functionally regulated by temperature.

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The authors declare no conflict of interest.

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Data deposition: Gene expression data reported in this paper have been deposited in the ArrayExpress database, <https://www.ebi.ac.uk/arrayexpress> (accession no. E-MTAB-5158).

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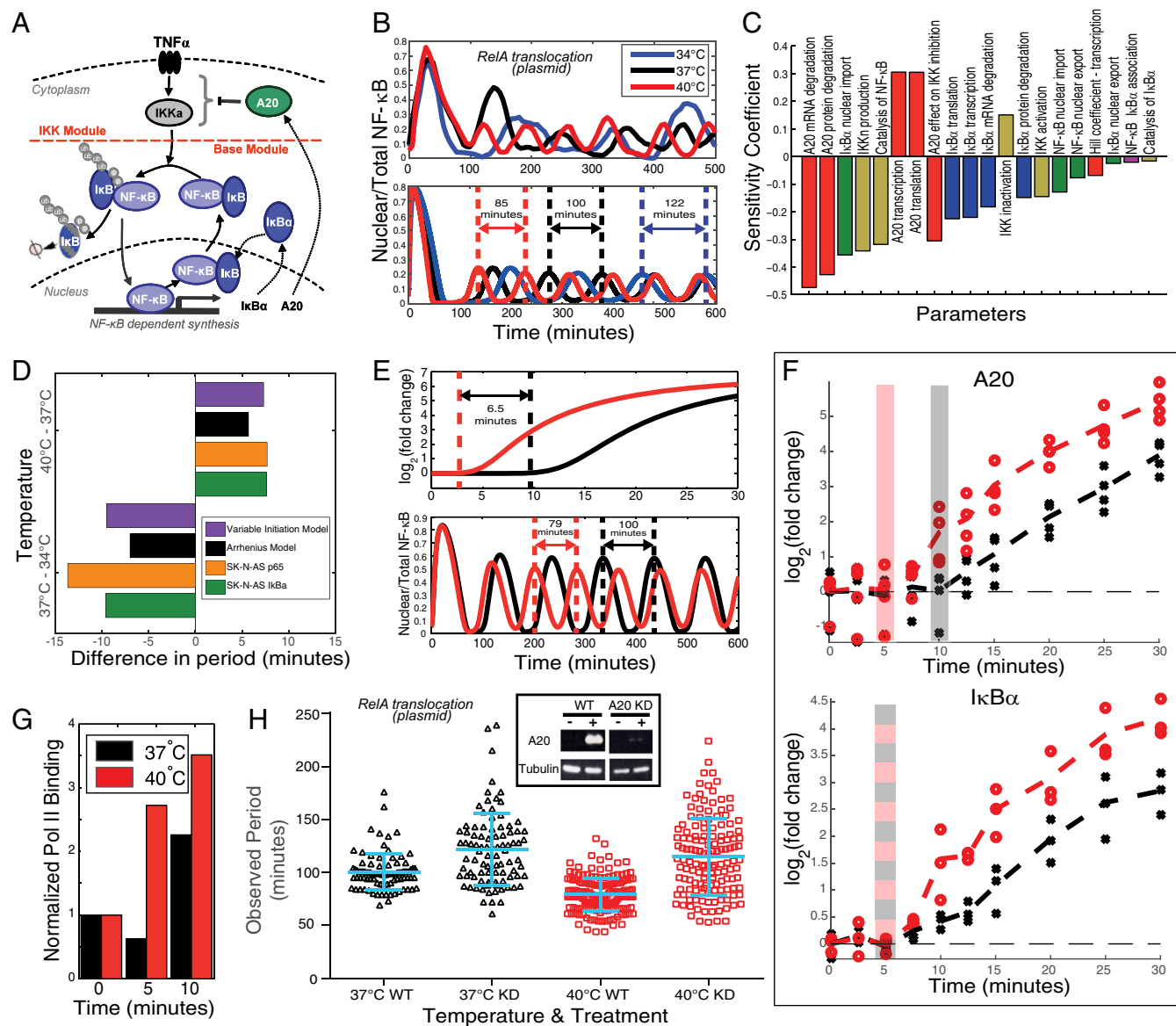


Fig. 2. Temperature dependence of the NF- κ B system depends on A20. (A) Diagram of the NF- κ B pathway showing the interactions between the main components incorporated into the model. (B) Example traces of RelA-DsRed_{XP} oscillations post-TNF α treatment at 34 °C, 37 °C, and 40 °C (Top), compared with simulations of single-cell trajectories from the NF- κ B model with parameters depending on temperature through the Arrhenius equation (Bottom). (C) Sensitivity analysis indicates that the model parameters that are most sensitive are linked to the synthesis and reactions of A20. (D) The experimentally observed change in the oscillation period per degree temperature below or above 37 °C, for SK-N-AS cells transiently expressing RelA-DsRed_{XP} (orange) or stably expressing I κ B α -eGFP (green), compared with the prediction of the original model (3) with adjusted parameters for temperature change (black), or as predicted by a modified model (purple) (for details, see *SI Appendix, SI Methods*). (E) The modified NF- κ B model incorporated a transcriptional delay of A20 of ~6.5 min (Top). This change was able to recapitulate the level of change in the observed period between 37 °C and 40 °C (Bottom). (F) RT-PCR analysis over the first 30 min post-TNF α treatment for A20 (Top) and I κ B α (Bottom) mRNA expression at 37 °C and 40 °C. Statistical analysis was used to determine the point of first up-regulation (*SI Appendix, SI Methods*) at 37 °C (gray shading) and 40 °C (red shading). (G) ChIP analysis of RNA polymerase II DNA binding to the A20 gene after continuous TNF α stimulation at 37 °C and 40 °C. (H) The effect of siRNA knockdown of A20 on NF- κ B oscillatory period at 37 °C and 40 °C (for scrambled siRNA control, see *SI Appendix, Fig. S5*); there was no significant difference ($P < 0.05$) between the knockdowns at these temperatures (121 ± 33 min and 116 ± 36 min, respectively). (Inset) Western blot showing the efficacy of the A20 siRNA knockdown with and without TNF α treatment. Throughout the figure, color denotes experimental and simulated temperature conditions: blue, 34 °C; black, 37 °C; and red, 40 °C.

We analyzed the model for simple structural changes, which might predict dynamics compatible with the experimentally observed oscillation dynamics. One such change incorporated a putative regulated (delayed) timing of A20 transcription (*SI Appendix, SI Methods 2*; see also data in Fig. 2 D and E). This modified model predicted that a putative 6.5-min-faster onset of A20 transcription at 40 °C, compared with 37 °C, was one simple structural change to the model that was able to produce dynamics consistent with the experimental observations (Fig. 2 E).

This prediction was initially supported by the microarray experiments and was further supported by analysis of a detailed qPCR time course of TNFAIP3/A20 mRNA expression following TNF α treatment at 37 °C and 40 °C (Fig. 2 F). In contrast, no similar difference was observed in the onset of expression of the other feedback inhibitors I κ B α (Fig. 2 F) or I κ B β (*SI Appendix, Fig. S3*). This suggested the possibility of a specific effect of temperature on A20 feedback kinetics. Quantification of bound RNA Polymerase II on the A20 gene using ChIP indicated

earlier transcription elongation at 40 °C, compared with 37 °C (Fig. 2G). Western blot analysis of A20 protein levels also showed a temperature-dependent change in the dynamics of A20 expression at the protein level (*SI Appendix*, Fig. S4). These observations therefore supported a temperature-dependent change in the timing of the onset of A20 expression that was compatible with the model prediction.

We found that siRNA-mediated A20 knockdown increased the period of TNF α -induced oscillations at both 37 °C and 40 °C (Fig. 2H and *SI Appendix*, Fig. S5). Treatment at either temperature in the A20-depleted cells gave the same period of ~120 min, indicating that the dynamics of the system became insensitive to temperature. These data support the hypothesis that temperature regulation of the A20 feedback loop is an important component in the control of the timing of the oscillatory dynamics of the NF- κ B system.

The effect of temperature on the gene expression response to TNF α was studied using microarrays (validated by RT-qPCR; *SI Appendix*, Fig. S6). Expression profiles of NF- κ B-dependent genes, induced by TNF α , have previously been categorized by the time at which they reached their peak level of expression after the cells were stimulated at 37 °C (15, 16). Here, our microarray data were both analyzed by the previously described Hao and Baltimore criteria, or newly classified into immediate, middle, or slow by the time (30, 130, and either 230 or 430 min, respectively, poststimulation) at which they first displayed a significant change from their initial expression (*SI Appendix*, Fig. S7). A set of immediate genes were up-regulated at 40 °C compared with 37 °C after 30 min, but, notably, were unaffected by temperature at later time points (Fig. 3A). The middle (Fig. 3B) and slow genes (Fig. 3C) were also predominantly unaffected, with occasional sporadic patterns of differential expression. Very few TNF α -responsive genes were significantly affected by temperature alone (6%; see *Dataset S1*). These data suggest temperature insensitivity of a majority of target genes, accompanied by the temperature-dependent regulation of the dynamics of a different subset of genes.

We categorized those genes that exhibited significant differential expression at 40 °C compared with 37 °C after TNF α stimulation by the time point at which this difference first occurred (denoted DE_{TEMP#}, where # is the time point), and subdivided them based on known NF- κ B targets or otherwise (κ B or non- κ B genes; *SI Appendix*, *SI Methods 4* and Table S2 and *Dataset S1*). Of the DE_{TEMP30} genes, all but two of the κ B genes were up-regulated, and approximately two-thirds of these more than doubled their expression (including TNFAIP3/A20; Fig. 3D, *Left*). At subsequent time points, the mRNA levels of κ B genes were similar at the two temperatures. The behavior of the corresponding non- κ B genes was significantly different at each time point ($P < 0.05$) as they were both up- and down-regulated at 30 min, and had differing expression at later time points (Fig. 3D, *Right*). Similarly, in the DE_{TEMP130} genes, we predominantly observed up-regulation at 130 min in κ B genes in comparison with the non- κ B genes, but there were no significant behavioral differences at subsequent times (Fig. 3E). However, the DE_{TEMP230} and DE_{TEMP430} genes generally showed temperature-dependent down-regulation, whether or not they were known κ B targets (Fig. 3F and G). Our principal observation was therefore the identification of a set of immediate NF- κ B-dependent genes that show early temperature-dependent up-regulation followed by subsequent temperature insensitivity.

We analyzed Gene Ontology (GO) categories (*SI Appendix*, *SI Methods 4*) of TNF α -dependent genes which were differentially expressed with respect to temperature (Fig. 3H and I). These were compared with the genes which showed a transcriptional response to TNF α alone at 37 °C (*SI Appendix*, Fig. S8). Increases were observed in the number of temperature-regulated

κ B (but not non- κ B) genes within important GO categories, including immune and inflammatory responses.

To further elucidate the functional effects of genes affected by temperature, we performed an analysis of their linkages in terms of protein interaction networks and signaling networks. Genes that showed significant temperature-dependent changes mapped to key cell signaling pathways involved in cytokine networks, cell repair and survival, and feedback control of the TNF α /NF- κ B system, as illustrated in Fig. 4. They included BIRC3/cIAP2, which is involved in feedback control of NF- κ B and apoptosis; IL1 α , which is strongly linked to the control of fever; GADD45B, which is involved in links to p53-dependent DNA repair and apoptosis control; MAP3K8 (TPL2), linked to the ERK/JNK pathways (all κ B genes); and Axin2, which is a key inhibitor of the Wnt signaling pathway. Several genes also showed linkages between the protein–protein interaction networks and key functional pathways (*SI Appendix*, Figs. S9–S11). Analysis of protein levels of some key genes showed that, for A20 (*SI Appendix*, Fig. S4), BIRC3/cIAP2, Traf1, and Axin2 (*SI Appendix*, Fig. S12), the observed changes in RNA expression were also observed at the protein level. Our work therefore suggests that temperature affects the pattern of NF- κ B-dependent gene expression in a highly controlled manner that enables precise and coordinated inflammatory and cell-fate responses to infection and stress, while leaving other functions unperturbed.

Discussion

We have found that changes in temperature alter both the oscillatory frequency of NF- κ B and the gene expression response to TNF α . As we predominantly observed an enhanced early response in the κ B genes, followed by unchanged expression levels at later time points, we suggest that the oscillatory and transcriptional effects of temperature are intertwined. We propose that the observed temperature insensitivity may constitute a form of temperature compensation in gene expression, which is a result of the interaction between the increased oscillation frequency and the generic effects of temperature on synthesis and degradation rates. This “temperature compensation” may imply a biological requirement that the basic inflammatory and immune response to pathogens must remain functional across the physiological temperature range. At the same time, the differential timing of expression of a specific group of κ B genes may play a key role in the modulation of cellular responses to cytokine stimulation at altered temperatures.

There may also be direct or indirect functional effects through genes classified as non- κ B genes, which may be differentially expressed at later time points. These non- κ B genes most likely include some NF- κ B-regulated genes where the direct role of NF- κ B has not yet been defined. With this in mind, one particularly interesting observation was the strong down-regulation at 40 °C compared with 37 °C of AXIN2 (for mRNA data, see *SI Appendix*, Fig. S6, and, for Western Blot, see *SI Appendix*, Fig. S12), a key Wnt pathway feedback repressor (17) (Fig. 4), and a known oscillator (18). These data may suggest temperature modulation of Wnt/NF- κ B cross-talk.

One of the κ B genes that showed temperature-sensitive dynamics of early onset gene expression was the key feedback inhibitor A20/TNFAIP3. We tested a mathematical model prediction that modulation of A20 might affect the sensitivity of the NF- κ B system to temperature. A20 knockdown removed the sensitivity to temperature between 37 °C and 40 °C, a temperature range relevant to the fever response. These data indicate a role for A20 in modulation of the inflammatory response in response to temperature changes. The other key feedback genes, I κ B α and I κ B ϵ , did not show the same consistent change in their TNF α -dependent expression dynamics at different temperatures (*SI Appendix*, Fig. S3). While it is possible that variation in the level of cell heterogeneity might represent an important modulator

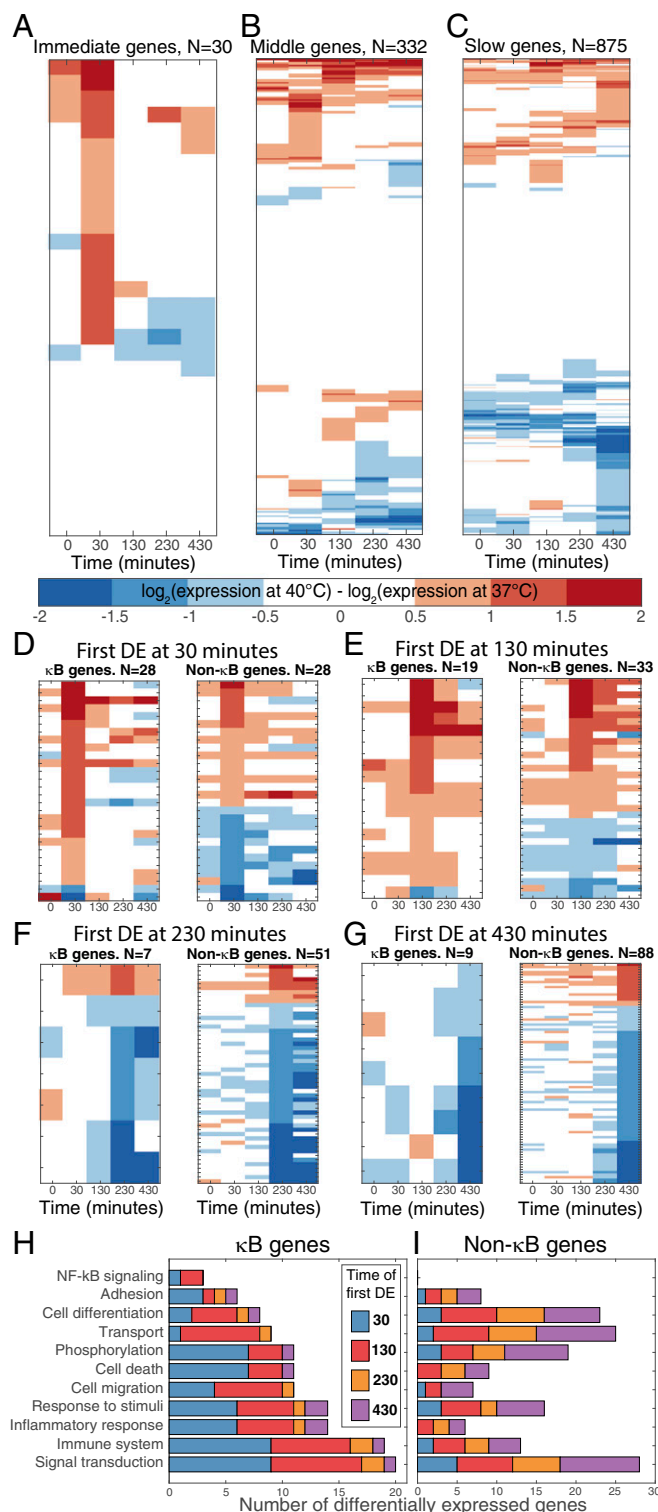


Fig. 3. Temperature change affects expression through early genes. Heatmaps showing the log difference in expression between 40°C and 37°C at 0, 30, 130, 230, and 430 min following treatment with $\text{TNF}\alpha$. A–C show how expression changes with temperature increase in the (A) immediate, (B) middle, and (C) slow genes, which were determined by the time of first differential transcription from baseline after $\text{TNF}\alpha$ treatment at 37°C (SI Appendix, SI Methods 4). Genes are ordered using hierarchical clustering of expression levels; corresponding gene names are given in Dataset S1. DE, differentially expressed. D–G show those genes with statistically significant differential transcription between the two temperatures after $\text{TNF}\alpha$ treatment. These are ordered by the first time point at which a significant dif-

ference was observed, and are further divided into κB genes (Left) and non- κB genes (Right). GO categories were established from these genes (SI Appendix, SI Methods 4), and the timing and number of genes that fell into each category are shown as separate bar charts for (H) κB and (I) non- κB genes, the names of which are given in SI Appendix, Table S2 and Dataset S1. Transcript variants are treated separately throughout.

of a cell population/tissue level inflammatory response (19), no consistent change in cell-to-cell dynamic heterogeneity was observed (SI Appendix, SI Methods 1), and the absence of temperature regulation of I $\kappa\text{B}\epsilon$ provides no evidence for a mechanism by which temperature may regulate cellular heterogeneity. Overall, these data suggest that A20 may therefore represent a specific target for therapeutic intervention.

Recent studies using an information theoretic approach have suggested that the ability of the NF- κB system to transmit information is relatively limited, enabling, at most, a binary decision (7, 20). Given the complex downstream roles assigned to NF- κB , such a limitation has seemed rather counterintuitive, and our results underscore this concern. These previous studies have focused on the mutual information between the cytokine level and the amount of transcription factor in the nucleus at a set of given times. They have not considered all aspects of the response, omitting key aspects, such as the dynamics, modification states (e.g., phosphorylation) of NF- κB subunits, interactions between these subunits, and the overall gene expression pattern. By studying stimulation, coupled to temperature perturbation, we now hypothesize that, when NF- κB dynamics and the gene expression profile are taken into account, the ability of NF- κB to transmit information may be significantly greater than has been previously suggested.

There has been a great deal of work suggesting that the inflammatory response may be regulated by temperature–fever (21). At febrile temperatures, NF- κB activity has been shown to be modulated by factors such as HSF1 to ensure correct regulation of the proinflammatory response (22). In our studies, we observed that temperature affects NF- κB dynamics at not only increased but also decreased temperatures (Fig. 1 and SI Appendix, Fig. S1), including temperatures well below those normally associated with a heat shock response. A number of studies have suggested that febrile temperatures can affect the expression of inflammatory cytokines. Rhinovirus infection of airway cells at lowered airway temperatures has been suggested to be due to a differential innate immune response at these lower temperatures (23, 24). In addition, animal housing temperature can have a dramatic effect on tumor formation and immune responses in laboratory mice (25). We speculate that the association of sleep pattern/circadian rhythms with NF- κB disruption (26), chronic inflammation (27), and cancer prognosis (28) could be due, in part, to altered sleep–wake temperature cycles (e.g., caused by shift work).

In conclusion, we show that small temperature changes can dramatically alter the dynamics of the NF- κB response to cytokine stimulation. This is mediated through the A20 feedback inhibitor and leads to specific dynamic changes in the expression of key NF- κB –dependent genes involved in the inflammatory response, as well as cross-talk with other important cell-fate-regulating pathways. Overall, these results suggest that temperature regulates the inflammatory response through modulation of NF- κB signaling and its downstream functions.

Methods

Cell Culture and Reagents. Experiments were performed on three cell models: human neuroblastoma SK-N-AS cells transfected with RelA-dsRedXP plasmid, SK-N-AS cells stably expressing an I $\kappa\text{B}\alpha$ -eGFP BAC as described previously (29, 30), and primary mouse adult fibroblasts (MAFs) cultured from ear

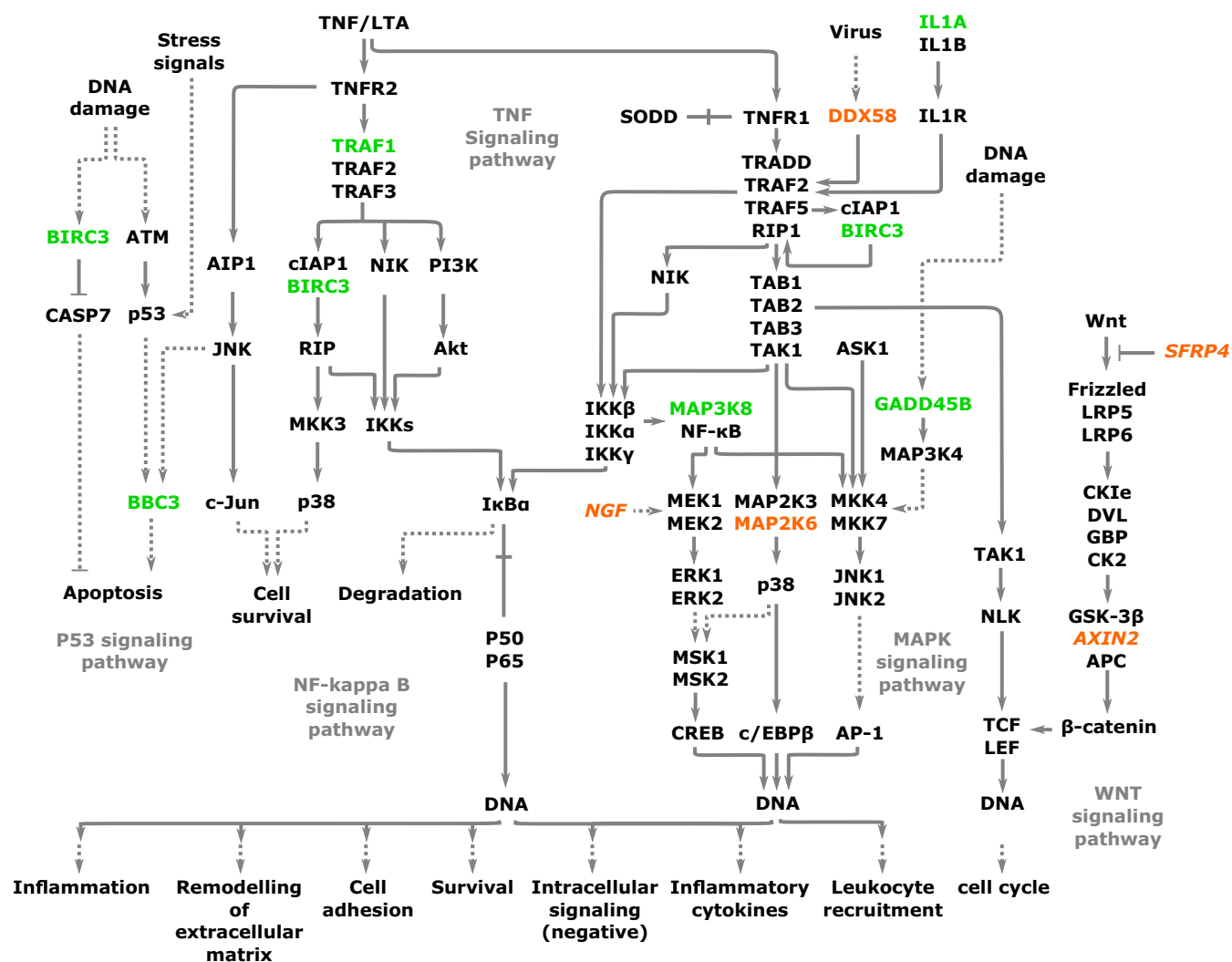


Fig. 4. Proteins derived from genes differentially expressed with temperature feature in important signaling pathways. Pathway analysis with the Kyoto Encyclopedia of Genes and Genomes (33) (KEGG) revealed where proteins derived from both κ B genes (green) and non- κ B genes (orange) are involved in key signaling pathways. Genes that were down-regulated at 40 °C compared with 37 °C are shown in *italics*; the remaining genes are up-regulated. Where gene and protein names differ, the gene name is given, for consistency. These interactions indicate that their differential expression of the associated genes could have important implications for future signal transduction through these pathways. To avoid bias, only interactions identified using KEGG are shown, leading to the omission of the roles of notable proteins such as A20 (TNFAIP3) in the NF- κ B signaling pathway, and NEDD4L in the Wnt pathway.

punches from transgenic mice expressing an RelA-DsRed_{XP} BAC (*SI Appendix*). SK-N-AS cells were cultured in Modified Eagles Medium supplemented with 1% nonessential amino acids (Sigma-Aldrich), and MAFs were cultured in Dulbecco Modified Eagles Medium. Both cell types were supplemented with 10% FBS (Gibco). Transient transfection was performed as described previously (4). For bulk cell experiments (ChIP, RT-PCR, microarray analysis, and siRNA), experimental dishes were transferred to a humidified 5% CO₂ incubator at 40 °C 1 h before the start of the time course.

siRNA Knockdown. SK-N-AS cells were plated at a density of 36,000 cells per dish. A20 was targeted using a pool of four ON-TARGET plus siRNAs (200 nM; J-009919-05, J-009919-06, J-009919-07, and J-009919-08; Dharmacon), or low GC content scrambled siRNA transfected into cells using the SK-N-AS transfection kit (AltoGen Biosystems) according to manufacturer's instructions. Cells were transfected, as described previously (3), 24 h before imaging.

Western Blot. Nitrocellulose membranes (Optitran BA-S85; Schleider & Schuell) were incubated overnight with primary antibodies against A20 (#59A426; Calbiochem), α -tubulin (#2144; Cell Signaling), Traf1 (#4715; Cell Signaling), c-IAP2 (#3130; Cell Signaling), or Axin2 (#2151; Cell Signaling) followed by HRP-conjugated secondary antibodies (Cell Signaling).

RT-qPCR. Cells were plated at a density of 500,000 cells per dish. Total RNA was extracted using the High Pure RNA Isolation kit (Roche), according to manufacturer's instructions, and quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific); 1 μ g of RNA was reverse-transcribed and amplified using the SuperScript VILO cDNA synthesis kit (Life Technologies). For RT-qPCR, cDNA was diluted 1:20 with RNase-free water, and amplified using the LightCycler 480 SYBR Green 1 Master Mix (Roche). Three biological replicates were performed. Relative quantification of gene expression was conducted using GAPDH expression level as an endogenous control. For sequences of primers, see *SI Appendix, SI Methods*.

ChIP Assays. SK-N-AS cells (3×10^6) were plated in 10-cm² dishes and incubated at 37 °C for 48 h. Dishes were incubated at the appropriate temperature 1 h before treatment with 10 ng TNF α . ChIP assays were performed as described previously (3) based on the protocol by Upstate Biotechnology. Immunoprecipitation was carried out using 5 μ g of either Anti-RNA polymerase II (Merck 05-623) or Anti-IgG antibodies (Cell Signaling #2729s). DNA was extracted and amplified by PCR as described previously (3). The following primer sequences were used: A20 gene forward GGTGTGGAGA-GCACAATGG, reverse CAGTGTGTATCGGTGCATGG, amplifying 160 bp of DNA. The qPCR was performed using LightCycler 480 SYBR Green 1 Master Mix (Roche).

Fluorescent Time-Lapse Imaging. Cells (SK-N-AS: 100,000; RelA-dsRedXP Primary MAFs: 25,000) were seeded in 35-mm glass coverslip-based dishes (Greiner) 20 h before imaging. Cells were transferred to the stage of a Zeiss Axiovert 200 equipped with an XL incubator (maintained at the appropriate temperature and 5% CO₂, in humid conditions), housed in temperature-controlled rooms at 26 ± 0.5 °C to aid accurate temperature control on microscopes; 10 ng/mL of TNF α was added to the dish 1 h after transfer onto the microscope, and images were captured every 5 min. Confocal microscopy was carried out as previously described (3) using a 40 \times Fluor NA 1.3 oil-immersion objective (Zeiss). Nuclear:cytoplasmic ratio of RelA-DsRed_{XP} or whole cell intensity of I κ B α -eGFP was calculated using Cell Tracker (31, 32). The timing of the second to sixth peaks (RelA-DsRed_{XP}) or troughs (I κ B α -eGFP) was recorded for each cell. Culture medium during imaging, microscope acquisition parameters, and imaging data quantification were as previously described (3).

Microarray Experiment. SK-N-AS cells were plated at a density of 500,000 cells per dish. Time-course experiments were carried out 48 h later. Cells were transferred to the two temperatures, 37 °C and 40 °C, for 1 h and then (at time 0) stimulated with 10 ng/mL of TNF α . Measurements were taken at 0, 30, 130, 230, and 430 min. Cells were lysed, and total RNA was extracted and quantified as above. The cDNA was obtained using the TransPlex Whole Transcriptome Amplification Kit and JumpStart Taq DNA polymerase (Sigma-Aldrich), with 300 ng of RNA starting material and 17 cycles of PCR amplification. The resulting cDNA was purified using the QIAquick PCR

purification kit (Qiagen). The cDNA was labeled and analyzed on NimbleGen 12 \times 135K Homo Sapiens Expression Arrays (100718 HG18 opt expr) by the Roche array service and subject to robust multiarray analysis normalization using the proprietary NimbleScan package.

Data Availability. All microarray data used in this study are available in the ArrayExpress database (<https://www.ebi.ac.uk/arrayexpress>) under accession number E-MTAB-5158.

Detailed methods are provided in *SI Appendix, SI Methods*.

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